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CURRENT PROTOCOLS IN MOLECULAR BIOLOGY

Published by Greene Publishing Associates and Wiley-Interscience

JOHN WILEY & SONS

New York • Chichester • Brisbane • Toronto • Singapore

Library of Congress Cataloging in Publication Data:

Current protocols in molecular biology.
1. Molecular biology—Technique. 2. Molecular biology—Laboratory manuals. I. Ausubel, Frederick M.

QH506.C87 1987 574.8'8'028 87-21033
ISBN 0-471-62594-9
ISBN 0-471-63248-1 (pbk.)

Printed in the United States of America

10 9 8 7 6 5 4 3

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INTRODUCTION

The application of molecular biology techniques to the analysis of complex genomes depends on the ability to prepare pure, high-molecular-weight DNA. This chapter begins with protocols for purification of genomic DNA from bacteria, plant cells, and mammalian cells. These protocols consist of two parts: a technique to gently lyse the cells and solubilize the DNA, followed by one of several basic enzymatic or chemical methods to remove contaminating proteins, RNA, and other macromolecules. The basic approaches described here are generally applicable to a wide variety of starting materials. A brief general protocol for further purifying and concentrating nucleic acids is also included.

Virtually all protocols in molecular biology require, at some point, fractionation of nucleic acids of different sizes. Gel electrophoresis has much greater resolution than alternative techniques such as density gradient centrifugation, and is the fractionation method of choice. Gel electrophoretic separations can be either analytical or preparative, and can involve fragments with molecular weights ranging from less than 1000 to more than 10 million. A variety of electrophoretic systems have been developed to accommodate such a large range of applications.

In general, the use of electrophoresis to separate nucleic acids is simpler than its application to resolve proteins. Discontinuous buffer systems are not necessary for nucleic acids, for example. Nucleic acids are uniformly negatively charged and, for double-stranded DNA, reasonably free of complicating structural effects that affect mobility. Although a detailed and quantitatively theoretical analysis of the process of electrophoresis has not been developed, a large amount of empirical study has identified a variety of important variables that affect migration of nucleic acids on gels. These include the conformation of the nucleic acid, the pore size of the gel, the voltage gradient applied, and the salt concentration of the buffer. The most basic of these variables is the pore size of the gel, which dictates the size of the fragments that can be resolved. In practice, this means that larger pore agarose gels are used to resolve fragments larger than 1000 bp and smaller pore acrylamide or sieving agarose gels are used for fragments smaller than 1000 bp. Sections II (large fragments) and III (small fragments) of this chapter describe analytical and preparative applications of such gels.

Frequently it is desirable to identify an individual fragment in a complex mixture that has been resolved by gel electrophoresis. This is accomplished by a technique termed Southern blotting (*UNIT 2.9*), in which the fragments are transferred from the gel to a filter and the fragment of interest is identified by hybridization with a labeled nucleic acid probe (usually radioactive). This approach is widely used to identify specific fragments in a digest of total genomic DNA.

Other commonly encountered applications of gel electrophoresis include resolution of single-stranded RNA or DNA. Polyacrylamide gels containing high concentrations of urea as a denaturant provide a very powerful system for resolution of short (<500 nucleotide) fragments of single-stranded DNA or RNA. Such gels can resolve fragments differing by only a single nucleotide in length, and are central to all protocols for DNA sequencing. A detailed description of such denaturing polyacrylamide gels is found in *UNIT 7.4*. Such gels are used for other applications requiring resolution of single-stranded fragments, particularly including the techniques for analyzing mRNA structure by S1 analysis (*UNIT 4.6*), ribonuclease protection (*UNIT 4.7*), or primer extension (*UNIT 4.8*). Denaturing polyacrylamide gels are also useful for preparative applications, such as small-scale purification of radioactive single-stranded probes and large-scale purification of synthetic oligonucleotides.

Resolution of relatively large single-stranded fragments (>500 nucleotides) can be accomplished using denaturing agarose gels. This is of particular importance to the analysis of mRNA populations by northern blotting and hybridization. A protocol for use of agarose gels containing formaldehyde in resolution of single-stranded RNA is presented in *UNIT 4.9*. The use of denaturing alkaline agarose gels for purification of labeled single-stranded DNA probes is described in *UNIT 4.6*.

Gels and Electric Circuits

Gel electrophoresis units are almost always simple electric circuits and can be understood using two simple equations that describe electricity. These equations underlie choices of voltage and buffers and allow understanding of factors, such as heating, which limit various applications of gels. Ohm's law, $V = IR$, states that the electric field, V (measured in volts), is proportional to current, I (measured in milliamps), times resistance, R (measured in ohms). When a given amount of voltage is applied to a simple circuit, a constant amount of current flows through all the elements and the decrease in the total applied voltage that occurs across any element is a direct consequence of its resistance. For a segment of a gel apparatus, resistance is inversely proportional to the cross-sectional area and inversely proportional to the ionic strength of the buffer. Usually the gel itself provides nearly all of the resistance in the circuit, since it has by far the smallest cross-sectional area. As a consequence, the voltage applied to the gel will be essentially the same as the total voltage applied to the circuit, as indicated by the power supply. For a given current, decreasing either the thickness of the gel (and any overlying buffer) or the ionic strength of the buffer will increase resistance and, consequently, increase the voltage gradient across the gel. This increases the electrophoretic mobility of the sample, which is directly proportional to voltage.

A practical upper limit to the voltage is usually set by the ability of the gel apparatus to dissipate heat. A second useful equation, $P = I^2R$, states that the power produced by the system, P (measured in watts), is proportional to the resistance times the square of the current. The power produced is manifested as heat, and any gel apparatus can dissipate only a particular amount of power without increasing the temperature of the gel. Above this point small increases in voltage can cause significant and potentially disastrous increases in temperature of the gel. It is very important to know how much power a particular gel apparatus can easily dissipate and to carefully monitor the temperature of gels run above that level.

Two practical examples illustrate applications of the two equations. The first involves the fact that the resistance of acrylamide gels increases somewhat during a run as ions related to polymerization are electrophoresed out of the gel. If such a gel is run at constant voltage, the current will increase with time and significant increases in power can occur. If an acrylamide gel is being run at high voltage, the power supply should be set to deliver constant power rather than constant voltage. The second situation is the case where there is a limitation in number of power supplies, but not gel apparati. A direct application of the first equation shows that the fraction of total voltage applied to each of two gels hooked up in series (one after another) will be proportional to the fraction of total resistance the gel contributes to the circuit. Two identical gels will each get 50% of the total voltage and power indicated on the power supply.

Finally, it should be noted that some electrophoretic systems employ lethally high voltages, and almost all are potentially hazardous. It is very important to use an adequately shielded apparatus, an appropriately grounded and regulated power supply, and most importantly, common sense when carrying out electrophoresis experiments.

PREPARATION OF GENOMIC DNA

This section begins with a protocol describing basic techniques for purifying and concentrating DNA samples (*UNIT 2.1*), followed by three similar protocols for purification of DNA from mammalian tissue (*UNIT 2.2*), plant tissue (*UNIT 2.3*), and bacteria (*UNIT 2.4*).

IMPORTANT NOTE: *The smallest amount of contamination of DNA preparations by recombinant phages or plasmids can be disastrous. Many person-years have been wasted reisolating previously cloned sequences that contaminated preparations of DNA used to create recombinant DNA libraries (see Hall, 1987, for an account of such a mistake), and many researchers have been embarrassed to find that the "extra" genes they found on their Southern blots were actually contaminating plasmid DNA. All materials used for preparation of plasmid or phage DNA should be kept separate from those used for preparation of genomic DNA, and disposable items should be used wherever possible. Particular care should be taken to avoid contamination of commonly used rotors.*

Purification and Concentration of DNA from Aqueous Solutions

This unit presents basic procedures for manipulating solutions of single- or double-stranded DNA through purification and concentration steps. These techniques are useful when proteins or solute molecules need to be removed from aqueous solutions, or when DNA solutions need to be concentrated for reasons of convenience. The basic protocol is appropriate for the purification of DNA at concentrations up to 1 mg/ml from small volumes (<0.4 ml) of aqueous solution. Additional procedures are presented for extracting and precipitating DNA from larger volumes and from dilute solutions, and for removing low-molecular-weight oligonucleotides and triphosphates. Finally, a support protocol is provided for buffering phenol for use in nucleic acid purification.

PHENOL EXTRACTION AND ETHANOL PRECIPITATION OF DNA

Materials

25:24:1 phenol/chloroform/isoamyl alcohol (with *buffered* phenol; see support protocol, p. 2.1.5)
3 M sodium acetate, pH 5.2
Ice-cold 100% ethanol
70% ethanol
TE buffer (*APPENDIX 2*)

Phenol extraction

1. Add an equal volume of phenol/chloroform/isoamyl alcohol to the DNA solution to be purified (0.1 to 0.4 ml in microcentrifuge tube).

   Solutions containing monovalent cations ≤0.5 M can be used. Extracting volumes of 50 to 100 µl is difficult; smaller volumes can be diluted to reach this lower limit.

   High salt concentrations can cause the inversion of the aqueous and organic phases. If this happens, the organic phase can be identified by its yellow color.

2. Vortex vigorously for 10 sec.

3. Spin 15 sec at room temperature in a microcentrifuge.

   Phases should be well separated. If DNA solution is viscous or contains a large amount of protein, spin longer (1 to 2 min).

4. Carefully remove the top (aqueous) phase containing the DNA using a 200-µl pipettor and transfer to a new tube.

   If starting with a small amount of DNA (< 1 µg), recovery can be improved by reextracting the organic phase with 100 µl TE buffer. This aqueous phase can be pooled with that from the first extraction.

5. If a white precipitate is present at the aqueous/organic interface, repeat steps 1 to 4.

Ethanol precipitation

6. In a 1.5-ml microcentrifuge tube add 1/10 vol of 3 M sodium acetate, pH 5.2, to the solution of DNA. Mix by vortexing briefly or by flicking the tube several times with a finger.

   If the solution contained a high concentration of NaCl or sodium acetate (0.3 to 0.5 M) prior to the phenol extraction step then no additional salt should be added. It is advisable to make appropriate dilutions in order to keep NaCl and sodium acetate concentrations below 0.5 M. If ethanol precipitation is not desirable, residual organic solvents can be removed by ether extraction. In this case, do not add salt and see the alternate procedures which follow.

7. Add 2 vol (calculated *after* salt addition) or more of ice-cold 100% ethanol. Mix by vortexing and place in crushed dry ice for 5 min or longer.

   This precipitation step can also be done in a −70°C freezer for at least 15 min or longer, or in a −20°C freezer for at least 30 min. A slurry of dry ice and ethanol may also be used, but tube labels are less often lost when crushed dry ice is used.

8. Spin 5 min in a fixed-angle microcentrifuge.

9. For small pellets (< 1 µg) aspirate off the ethanol supernatant with a pipetting device such as a Pasteur pipet or pipettor. This is best accomplished by drawing off liquid from the side of the tube opposite that which the DNA precipitate was pelleted against. Start at the top and move downward as the liquid level drops.

   For large pellets the supernatant can simply be poured off.

10. Add 1 ml of 70% ethanol (room temperature). Invert the tube several times and spin in microcentrifuge as before.

    If the DNA molecules being precipitated are very small (<200 bases) use 95% ethanol at this step.

11. Aspirate off the supernatant as before.

    The DNA pellet will not stick well to the walls of the tube after the 70% ethanol wash and care must be taken to avoid aspirating the pellet out of the tube.

12. Dry the pellet in a desiccator under vacuum or in a Speedvac evaporator (Savant).

13. Dissolve the dry pellet in an appropriate volume of water if it is going to be used for further enzymatic manipulations requiring specific buffers. Dissolve in TE buffer if it is going to be stored indefinitely.

    DNA pellets will not dissolve well in high-salt buffers. To facilitate resuspension keep the DNA concentration of the final solution at < 1 mg/ml.

    If DNA is resuspended in a volume of TE buffer or water to yield a DNA concentration of < 1 mg/ml, small quantities (< 25 µg) of precipitated plasmids or restriction fragments should dissolve quickly upon gentle vortexing or flicking of the tube with a finger. However, larger quantities of DNA may require vortexing and brief heating (5 min at 65°C) to resuspend. High-molecular-weight genomic DNA may require one to several days to dissolve and should not be vortexed to avoid shearing,

particularly if it is to be used for cosmid cloning or other applications requiring high-molecular-weight DNA. Gentle shaking on a rotating platform or a rocking apparatus is recommended.

Purification and Concentration of RNA

All procedures are identical for purification of RNA except that 2.5 vol ethanol should be routinely used for precipitation (step 7). It is essential that all water used directly or in buffers be treated with diethylpyrocarbonate (DEPC) to inactivate RNase (see *UNIT 4.1*, reagents and solutions, for instructions).

Dilute Solutions of DNA

When DNA solutions are dilute (< 10 µg/ml) or when < 1 µg of DNA is present, increase the ratio of ethanol to aqueous volume to 3:1 and extend the time on dry ice to 30 min (step 7). Carry out microcentrifugation in a cold room and spin 15 min to ensure the recovery of DNA from these solutions.

Nanogram quantities of labeled or unlabeled DNA can be efficiently precipitated by the use of carrier nucleic acid. A convenient method is to add 10 µg of commercially available tRNA from *E. coli*, yeast, or bovine liver to the desired DNA sample. The DNA will be coprecipitated with the tRNA. The carrier tRNA will not interfere with most enzymatic reactions, but will be phosphorylated efficiently by polynucleotide kinase and should not be used if this enzyme will be used in subsequent radiolabeling reactions.

Recovery of small quantities of short DNA fragments and oligonucleotides can be enhanced by adding magnesium chloride to a concentration of < 10 mM before adding ethanol. However, DNA precipitated from solutions containing >10 mM magnesium or phosphate ions is often difficult to redissolve and such solutions should be diluted prior to ethanol precipitation.

DNA in Large Aqueous Volumes (> 0.4 to 10 ml)

Larger volumes can be simply scaled up using the same procedure. For the phenol extraction steps, use tightly capped 15- or 50-ml polypropylene tubes. (Polystyrene tubes cannot withstand the phenol/chloroform mixture.) Perform centrifugation steps at room temperature at speeds not exceeding 2500 rpm for 5 min. The ethanol precipitate should be centrifuged in thick-walled Corning glass test tubes (15- or 30-ml capacity) for 15 min in fixed-angle rotors at 10,000 rpm, 1°C. Glass tubes should be siliconized (see *APPENDIX 3*) to facilitate recovery of small amounts of DNA (< 10 µg).

Concentration of DNA Using Butanol

Water molecules (but not DNA or solute molecules) can be removed from aqueous solutions by extraction with *sec*-butanol (2-butanol). This procedure is useful for reducing volumes or concentrating dilute solutions.

1. Add an equal volume of *sec*-butanol to the sample (in a polypropylene tube) and mix well by vortexing or by gentle inversion if the DNA is of high molecular weight.
2. Centrifuge at room temperature for 5 min at 2500 rpm or in a microcentrifuge for 10 sec.
3. Remove and discard the upper (*sec*-butanol) phase.
4. Repeat steps 1 to 3 above until the desired volume of aqueous solution is obtained.
5. Extract the lower, aqueous phase with 25:24:1 phenol/chloroform/isoamyl alcohol

and ethanol precipitate as in steps 1 to 13 of the basic protocol, or remove *sec*-butanol by two ether extractions as described below.

Addition of too much sec-butanol can result in complete loss of the water phase into the sec-butanol layer. If this happens, add 1/2 vol of water back to the sec-butanol, mix well, and spin. The DNA can be recovered in this new, aqueous phase and can be further concentrated with smaller amounts of sec-butanol.

The salt concentration will increase in direct proportion to the volume decrease. The DNA can be precipitated with ethanol to readjust the buffer conditions.

Removal of Residual Phenol, Chloroform, or Butanol by Ether Extraction

DNA solutions that have been purified by extraction with phenol and chloroform, or concentrated with *sec*-butanol, can often be used without ethanol precipitation for enzymatic manipulations or in gel electrophoresis experiments if the organic solvents are removed by extraction with ether. Traces of ether are subsequently removed by evaporation. This procedure is useful only if the solute concentrations in the starting solution are compatible with what is needed in later steps. It is quite useful in purifying high-molecular-weight DNA since mechanical shearing of large nucleic acid molecules can occur during precipitation with ethanol.

CAUTION: *Ether is highly flammable and its vapors can cause drowsiness. All manipulations with ether should be carried out in a well-ventilated fume hood.*

1. Mix diethyl ether with an equal volume of water or TE buffer in a polypropylene tube. Vortex vigorously for 10 sec and let the phases separate.

   Ether is the top phase.

2. Add an equal volume of ether to the DNA sample. Mix well by vortexing or by gentle inversion if the DNA is of high molecular weight.

3. Spin in a microcentrifuge for 5 sec or let the phases separate by setting upright in a test tube rack.

4. Remove and discard the top (ether) layer.

5. Repeat steps 2 to 4 above.

6. Remove ether by leaving sample open under hood for 15 min (small volumes, 100 µl), or under vacuum for 15 min (larger volumes).

   The DNA solution will be free of organic solvents and will have solute concentrations that are roughly 3/4 of those that were in the aqueous solution before phenol extraction (solute concentrations are lowered in the two phenol/chloroform/isoamyl alcohol extractions steps).

Precipitation of DNA Using Isopropanol

In general, 1/2 vol isopropanol can substitute for a given volume of ethanol in precipitations. This allows precipitation from a starting volume of 0.7 ml in a single microcentrifuge tube, for example. Isopropanol is less volatile than ethanol and takes longer to remove by evaporation. Some salts are less soluble in isopropanol (compared to ethanol) and will be precipitated along with nucleic acids. Most of these unwanted ions can be removed by washing the pellets with 70% ethanol.

Removal of Low-Molecular-Weight Oligonucleotides and Triphosphates by Ethanol Precipitation

Small single- or double-stranded oligonucleotides (less than ~30 bp) and unincorporated nucleotides used in radiolabeling or other DNA modification reactions can be effec-

tively removed from DNA solutions by two rounds of ethanol precipitation in the presence of ammonium acetate. This approach is not sufficient to completely remove large quantities of linkers as used in cloning procedures (*UNIT 3.16*).

1. Add an equal volume of 4 M ammonium acetate to the reaction.
2. Add 2 vol (calculated *after* salt addition) of ice-cold ethanol.
3. Set tube in crushed dry ice for 5 min.
4. Spin in microcentrifuge 5 min at room temperature.
5. Remove supernatant and redissolve pellet in 100 µl TE buffer.
6. Repeat steps 1 to 5 above.

   Reprecipitation is required particularly if the DNA solution from step 1 contained Mg^{++} or other divalent or polyvalent cations that facilitate precipitation of oligonucleotides.
7. Add 1 ml of 70% ethanol to tube and invert several times.
8. Spin in microcentrifuge 5 min at room temperature.
9. Dry pellet as in basic protocol (step 12).

   While the removal of unincorporated nucleoside triphosphates, reaction products, and small oligonucleotides is effective, it is not absolute and the procedure should not be used in purifying DNA away from these small molecules prior to detailed biochemical or analytical studies.

BUFFERING PHENOL

SUPPORT PROTOCOL

For some purposes, fresh liquified phenol (88% phenol) can be used without further purification. However, for purification of DNA prior to cloning and other sensitive applications, phenol must be redistilled before use, since the oxidation products of phenol can damage and introduce breaks into nucleic acid chains. Redistilled phenol for use in nucleic acid purification is commercially available. Regardless of the source, the phenol must be buffered before use.

CAUTION: *Phenol can cause severe burns to skin and damage clothing. Gloves, safety glasses, and a lab coat should be worn whenever working with phenol, and all manipulations should be carried out in a fume hood. A glass receptacle should be available exclusively for disposing of used phenol and chloroform.*

Materials

8-hydroxyquinoline
Liquified phenol
50 mM Tris base (unadjusted pH ~10.5)
50 mM Tris·Cl, pH 8
Chloroform
Isoamyl alcohol

1. Add 0.5 g of 8-hydroxyquinoline to a 2-liter glass beaker containing a stir bar.
2. Gently pour in 500 ml of liquified phenol or melted crystals of redistilled phenol (melt in a water bath at 65°C).

   The phenol will turn yellow due to the 8-hydroxquinoline, which is added as an antioxidant.
3. Add 500 ml of 50 mM Tris base.

4. Stir 10 min at low speed with magnetic stirrer at room temperature. Keep beaker covered with aluminum foil.
5. Let phases separate at room temperature.
6. Gently decant the top (aqueous) phase into a suitable waste receptacle. Remove what cannot be decanted with a 25-ml glass pipet and a suction bulb.
7. Add 500 ml of 50 mM Tris·Cl, pH 8.
8. Repeat steps 4 to 7 (i.e., two successive equilibrations with 500 ml of 50 mM Tris·Cl, pH 8).

   The pH of the phenol phase can be checked with indicator paper and should be 8. If it is not, repeat steps 3 to 7 until it is.
9. Add 250 ml of 50 mM Tris·Cl, pH 8, or TE buffer and store at 4°C in brown glass bottles or clear glass bottles wrapped in aluminum foil.
10. For use in DNA purification procedure, mix 25 vol phenol (bottom yellow phase of stored solution) with 24 vol chloroform and 1 vol isoamyl alcohol.

    Phenol prepared with 8-hydroxyquinoline as an antioxidant can be stored at 4°C for up to 2 months.

COMMENTARY

Background Information

It is often necessary to purify and/or concentrate a solution of DNA prior to further enzymatic manipulations or analytical studies. This is important, for example, when DNA-modifying enzymes need to be removed prior to modification with a second enzyme or when DNA preparations contain contaminants that inhibit restriction enzyme digestion or other DNA modification reactions. The most commonly used method for deproteinizing a solution of DNA is extraction with phenol, which efficiently denatures proteins and probably dissolves denatured protein (Kirby, 1957). Chloroform is also a useful protein denaturant with somewhat different properties. Chloroform stabilizes the rather unstable boundary between an aqueous phase and a pure phenol layer. The use of the mixture also reduces the amount of aqueous solution retained in the organic phase (compared to a pure phenol phase) in order to maximize the yield (Penman, 1966; Palmiter, 1974). Isoamyl alcohol is added to prevent foaming of the mixture upon vortexing and to aid in the separation of the organic and aqueous phases (Marmur, 1961). The denatured protein forms a layer at the interface between the aqueous and organic phases and is thus isolated from the bulk of the DNA in the aqueous layer. This procedure is rapid, inexpensive, and easy to perform on multiple samples.

Ethanol precipitation is useful for concentrating DNA solutions and for removing residual phenol and chloroform from the deproteinized aqueous solution. It is also useful for providing DNA that is relatively free of solute molecules when buffer conditions need to be changed in going from one modification reaction to another. In the presence of relatively high (0.1 to 0.5 M) concentrations of monovalent cations, ethanol induces a structural transition in nucleic acid molecules which causes them to aggregate and precipitate from solution (Eickbush and Moudrianakis, 1978). However, since most salts and small organic molecules are soluble in 70% ethanol, ethanol precipitation and washing of the pellet will effectively desalt DNA. While sodium chloride, sodium acetate, and ammonium acetate are each capable of inducing precipitation, it is more difficult to remove sodium chloride due to lower solubility in 70% ethanol and its use is discouraged.

Critical Parameters

Oxidation products of phenol can damage nucleic acids and only redistilled phenol should be used. To ensure complete deproteinization, extractions should be repeated until no protein precipitate is present at the aqueous/organic interface.

In general, alcohol precipitation of nucleic acids requires the presence of at least 0.1 M monovalent cation in the starting aqueous solution. Precipitation from solutions of low concentrations of nucleic acids requires cooling to low temperatures to give good recovery. Precipitation of nucleic acids at high concentrations (>0.25 mg/ml after addition of ethanol) is very rapid at room temperature. Formation of a visible precipitate after adding alcohol and mixing well indicates complete precipitation and no chilling or further incubation is needed.

Anticipated Results

These procedures should result in virtually complete removal of proteins and quantitative recovery of nucleic acids. However, sequential extractions or precipitations require care and attention to detail to prevent accumulation of small losses at each step. It is particularly important to carefully recover the aqueous phase and reextract the organic phase to ensure full recovery of small amounts of DNA from phenol/chloroform extractions.

Time Considerations

Approximately 90 min should be allowed for carrying out steps 1 through 12 of the basic protocol on 12 DNA samples in microcentrifuge tubes. Buffering of phenol should be started at least 1 hr before the equilibrated phenol is needed.

Nucleic acids should not be left in the presence of phenol, but can be left indefinitely precipitated in alcohol or dried after precipitation.

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Preparation of Genomic DNA from Mammalian Tissue

BASIC PROTOCOL

Tissue is rapidly frozen and crushed to produce readily digestible pieces. The processed tissue is placed in a solution of proteinase K and sodium dodecyl sulfate and incubated until most of the cellular protein is degraded. The digest is deproteinized by successive phenol/chloroform/isoamyl alcohol extractions, recovered by ethanol precipitation, and dried and resuspended in buffer.

Materials

Liquid nitrogen
Digestion buffer
Ice-cold phosphate-buffered saline (PBS; *APPENDIX 2*)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)
7.5 M ammonium acetate
100% ethanol
70% ethanol
TE buffer, pH 8 (*APPENDIX 2*)

Cell preparation

Beginning with whole tissue

1. As soon as possible after excision quickly mince tissue and freeze in liquid nitrogen.

   If working with liver, remove the gallbladder, which contains high levels of degradative enzymes.

2. Starting with between 200 mg and 1 g, grind tissue with a mortar and pestle, or crush with a hammer to a fine powder.

   Keep the tissue fragments.

3. Suspend the powdered tissue in 1.2 ml digestion buffer per 100 mg tissue. There should be no clumps.

Beginning with tissue culture cells

1. Spin suspension cultures out of their serum-containing media. Trypsinize adherent cells and collect cells from the flask. Centrifuge 5 min at 1500 rpm and discard supernatant.

2. Resuspend cells with 1 to 10 ml ice-cold PBS. Centrifuge 5 min at 1500 rpm and discard supernatant. Repeat this resuspension and centrifugation step.

3. Resuspend cells in 1 vol digestion buffer. For $< 3 \times 10^7$ cells use 0.3 ml digestion buffer. For larger numbers of cells use 1 ml digestion buffer/10^8 cells.

Cell lysis and digestion

4. Incubate the samples with shaking at 50°C for 12 to 18 hr in tightly capped tubes.

   The samples will be viscous. After 12 hr incubation the tissue should be almost indiscernible, a sludge should be apparent from the organ samples, and tissue culture cells should be relatively clear.

Extraction of nucleic acids

5. Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol.

   CAUTION: *phenol is extremely caustic.*

6. Centrifuge 10 min at 10,000 rpm in a swinging bucket rotor.

If the phases do not resolve well, add another volume of digestion buffer and repeat the centrifugation.

If there is a thick layer of white material at the interface between the phases, repeat the organic extraction.

Purification of DNA

7. Transfer the aqueous (top) layer to a new tube and add 1/2 vol of 7.5 M ammonium acetate and 2 (original) vol of 100% ethanol. The DNA should immediately form a stringy precipitate. Recover DNA by centrifugation at 5000 rpm for 2 min.

This brief precipitation in the presence of high salt reduces the amount of RNA in the DNA. For long-term storage it is convenient to leave the DNA in the presence of ethanol.

If very high-molecular-weight DNA is needed, remove organic solvents and salt from the DNA by at least two dialysis steps against at least 100 vol TE buffer. Because of the high viscosity of the DNA, it is necessary to dialyze for a total of at least 24 hr.

8. Rinse the pellet with 70% ethanol. Decant ethanol and air dry the pellet.

It is important to rinse well to remove residual salt and phenol.

9. Resuspend DNA in TE buffer until dissolved. DNA may be shaken gently at room temperature or at 65°C for several hours to facilitate solubilization.

If necessary, residual RNA can be removed at this step by adding 0.1% sodium dodecyl sulfate (SDS) and 1 μg/ml DNase-free RNase and incubating 1 hr at 37°C, followed by organic extraction and ethanol precipitation, as above.

10. Store at 4°C; ~1 mg/ml DNA is a convenient working concentration. From 1 g mammalian cells, ~2 mg DNA can be expected.

REAGENTS AND SOLUTIONS

Digestion buffer

100 mM NaCl
10 mM Tris·Cl, pH 8
25 mM EDTA, pH 8
0.5% sodium dodecyl sulfate
0.1 mg/ml proteinase K

The proteinase K is labile and must be added fresh with each use.

COMMENTARY

Background Information

There are a number of different procedures for the preparation of genomic DNA. They all start with some form of cell lysis, followed by deproteinization and recovery of DNA. The main differences between various approaches lie in the extent of deproteinization and in molecular weight of the DNA produced. The isolation procedure described here is relatively brief and relies on the powerful proteolytic activity of proteinase K combined with the denaturing ability of the ionic detergent SDS. Use of proteinase K for DNA purification was described by Gross-Bellard et al. (1972) and Enrietto et al. (1983). EDTA is included in the digestion buffer to inhibit DNases.

Critical Parameters

To minimize the activity of endogenous nucleases, it is essential to rapidly isolate, mince, and freeze tissue. Tissue culture cells

should be cooled and washed quickly. As soon as the tissue is frozen or the tissue culture cells are added to the lysis buffer, DNA is protected from action of nucleases throughout this protocol. It is important that the tissue be well dispersed and not left in large lumps to permit rapid and efficient access to proteinase K and SDS.

It is crucial to generate very high-molecular-weight DNA for construction of phage (> 60 kb) or cosmid (>120 kb) genomic libraries. Two main precautions should be taken to maximize molecular weight: (1) minimize shearing forces by gentle (but thorough) mixing during extraction steps, and (2) after the extraction, remove organic solvents and salt from the DNA by dialysis, rather than by ethanol precipitation.

The absence of both cellular proteins and proteinase K in the final DNA solution is important for susceptibility of the genomic DNA to restriction enzyme action; therefore, care should be exercised in deproteination. To remove protein completely it may be necessary to repeat the proteinase K digestion. In general, highly pure DNA has an A_{260} to A_{280} ratio > 1.8, while 50% protein/50% DNA mixtures have OD_{260}/OD_{280} ratios of ~1.5.

Troubleshooting

Failure of the organic phase to separate cleanly from the aqueous phase is generally due to a very high concentration of DNA and/or cellular debris in the aqueous phase. Dilution with more digestion buffer and reextraction can remedy this problem.

Upon addition of the room-temperature ethanol to the extracted DNA solution, the DNA should precipitate in long, stringy fibers. If there is no precipitate or if the precipitate is flocculent, the DNA is either degraded or not purified away from cellular debris. Improper handling of the tissue before digestion or too much tissue in the digestion reaction are possible causes of such problems.

Anticipated Results

Approximately 2 mg DNA should be obtained from 1 g tissue or 10^9 cells. The DNA should be at least 100 kb long and should be digestible with restriction enzymes.

Time Considerations

This protocol involves effort on 2 days: tissue preparation on the first day followed by overnight lysis, and extraction/precipitation on the second day. Actual time spent on the procedure, however, will be less than 1 hr each day. The DNA can be stored indefinitely in the presence of ethanol, or at −20°C.

Literature Cited

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Gross-Bellard, M., Oudet, P., and Chambon, P. 1972. Isolation of high-molecular-weight DNA from mammalian cells. *Eur. J. Biochem.* 36:32.

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Preparation of Genomic DNA from Plant Tissue

BASIC PROTOCOL

Plant cells are lysed by the detergent Sarkosyl and the lysates digested with proteinase K. After clearing the insoluble debris from the lysate the nucleic acids are precipitated and the DNA purified on a cesium chloride density gradient.

Materials

Cold, sterile H_2O
Liquid nitrogen
Extraction buffer
10% (wt/vol) *N*-lauryl sarcosine (Sarkosyl)
Isopropanol
TE buffer (*APPENDIX 2*)
Cesium chloride
10 mg/ml ethidium bromide
CsCl-saturated isopropanol
Ethanol
3 M sodium acetate, pH 5.2

Beckman JA-14 rotor
5-ml quick-seal ultracentrifuge tubes
Beckman VTi80 rotor
15-G needle and syringe

Tissue preparation

1. Harvest 10 to 50 g fresh plant tissue. Plants may be placed in the dark for 1 to 2 days prior to harvest to reduce the starch content in the tissues.

   Younger plants are the preferred source of tissue since they have a lower polysaccharide content.

2. Rinse tissue with cold sterile water to remove adhering debris and blot dry.

3. Freeze tissue with liquid nitrogen and grind to a fine powder in a mortar and pestle.

   Keep the tissue frozen throughout this procedure by occasionally adding liquid nitrogen.

Cell lysis and digestion

4. Transfer frozen powder to a 250-ml centrifuge bottle and immediately add extraction buffer. Generally 2 to 4 ml extraction buffer is added per g fresh plant tissue. Stir gently to disperse tissue.

5. Add an appropriate volume of 10% (wt/vol) Sarkosyl to achieve a final concentration of 1%.

   It is important to add the Sarkosyl after *the tissue is resuspended in the extraction buffer. If Sarkosyl is included in the extraction buffer, premature lysis of the plant cells will interfere with tissue dispersal and lead to unwanted shearing of the DNA.*

6. Incubate 1 to 2 hr at 55°C.

   The lysate should be clear (and green) and slightly viscous. From this point on the solutions should be handled gently to reduce shearing of the DNA—do not vortex or mix vigorously.

7. Centrifuge lysate 10 min in a Beckman JA-14 rotor at 6000 rpm, 4°C, to pellet debris. Save the supernatant and centrifuge again if necessary to remove undigested debris.

Precipitation of DNA

8. Add 0.6 vol isopropanol to the supernatant and gently mix. A nucleic acid precipitate should be visible; if not, place at −20°C for 30 min.

9. Centrifuge 15 min in a Beckman JA-14 rotor at 8000 rpm (7500 × *g*), 4°C. Discard supernatant.

   Do not let the nucleic acid pellet dry or it will become extremely difficult to get into solution.

Purification of DNA

10. Resuspend pellet in 9 ml TE buffer. Incubate at 55°C to aid resuspension if necessary. Add 9.7 g of solid CsCl and mix gently until dissolved.

11. Incubate lysates on ice for 30 min. Centrifuge 10 min at 8000 rpm, 4°C.

    This clearing spin removes some of the insoluble debris remaining in the lysate. In addition, a small separate phase may form on the top of the solution after centrifugation; this is due to residual Sarkosyl in the lysate. Collect the supernatant but discard the Sarkosyl phase on the top.

12. Add 0.5 ml of 10 mg/ml ethidium bromide and incubate on ice for 30 min.

    CAUTION: *Ethidium bromide is a mutagen. Be careful and wear gloves.*

13. Centrifuge 10 min at 8000 rpm, 4°C.

    A large RNA pellet should form. At this point much of the lysate's unwanted constituents (RNA, protein, and carbohydrates) has been removed.

14. Transfer the supernatant to two 5-ml quick-seal ultracentrifuge tubes and seal tubes.

    Make sure tubes are full, balanced, and well sealed.

15. Centrifuge 4 hr in a Beckman VTi80 rotor at 80,000 rpm, 20°C, or overnight at 60,000 rpm, 20°C.

16. Collect DNA band using a large-bore needle (15-G) and syringe. To do this a hole is first punched into top of tube with needle and DNA band is removed after inserting the collecting needle/syringe through tube wall directly below the band.

    This operation is identical to that used during plasmid purification, except that only one band should be visible. This step is illustrated in UNIT 1.7.

    It may be necessary to visualize the DNA with UV illumination. If so, wear UV protective glasses or a face shield. In any case, an effort should be made to minimize exposure of gradient to visible light thereby reducing nicking of DNA caused by ethidium bromide.

17. Remove the ethidium bromide by repeatedly extracting the collected DNA with isopropanol that has been equilibrated over a CsCl-saturated aqueous phase.

18. Add 2 vol water and 6 vol ethanol to the DNA solution and mix. Incubate 1 hr at −20°C. Centrifuge 10 min at 8000 rpm (7500 × *g*), 4°C.

    The DNA may precipitate immediately as a single white mass, which can be collected using a Pasteur pipet with a hook introduced at the tip or by a brief centrifugation.

19. Resuspend pellet in TE buffer and reprecipitate DNA by adding 1/10 vol of 3 M sodium acetate and 2 vol ethanol. Incubate at −20°C if precipitate is not visible and collect DNA by centrifugation.

20. Briefly air dry the final pellet and resuspend in TE buffer.

REAGENTS AND SOLUTIONS

Extraction buffer

100 mM Tris·Cl, pH 8
100 mM EDTA
250 mM NaCl
100 µg/ml proteinase K

COMMENTARY

Background Information

This protocol is an adaptation of common DNA isolation procedures: cell lysis by detergent, protease treatment, and CsCl gradient purification. Because whole cells are lysed, the DNA purified using this protocol will correspond to both the nuclear genome and cytoplasmic (mitochondrial and chloroplast) genomes. Methods for purifying nuclear DNA—free of plastid and mitochondrial DNA contamination—have been described by Watson and Thompson (1986). In addition, a miniprep protocol for isolation of total plant DNA has been described by Dellaporta et al. (1983); the miniprep protocol is similar to the protocol described here, omitting the CsCl gradient purification.

Critical Parameters

The aim of any genomic DNA prep is to isolate DNA of high molecular weight and sufficient purity. Two factors affect the size of the DNA isolated: shear and nuclease activity. As noted in the protocol, treat the lysates gently to reduce shear forces. In order to reduce nuclease activity (plant cells are rich in nucleases!) freeze the tissue quickly and thaw only in the presence of extraction buffer containing detergent and a high concentration of EDTA.

Plant DNA isolated using this protocol should be in the range of 50 kb in length which is quite acceptable for most applications. It has been our experience (working with *Arabidopsis*) that DNA isolated using this protocol digests well with restriction enzymes and can be ligated efficiently into cloning vectors. However, in some cases it may be necessary to modify the steps in order to reduce polysaccharide contamination, which is the most common problem affecting plant DNA purity. These carbohydrates, which are difficult to separate from the DNA itself, inhibit many enzymes commonly used in cloning procedures. The recommended procedure for polysaccharide removal is chloroform extraction of lysates in the presence of 1% CTAB (cetyltrimethylammonium bromide) and 0.7 M NaCl as described by Murray and Thompson (1980) and presented in the protocol for bacterial DNA purification, *UNIT 2.4*.

Anticipated Results

Yields should be in the range of 10 to 40 µg DNA (50-kb length) per gram fresh plant tissue. The isolated DNA digests well with restriction enzymes and can be ligated efficiently into cloning vectors.

Time Considerations

Approximately 4 to 6 hr are required to work through the protocol to the point where the lysate is loaded onto the CsCl gradient. The gradient can be spun overnight at 60,000 rpm or at a higher speed (80,000 rpm) for 4 hr. Approximately 3 to 4 hr will be required to process the banded DNA.

Literature Cited

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Preparation of Genomic DNA from Bacteria

BASIC PROTOCOL

MINIPREP OF BACTERIAL GENOMIC DNA

Bacteria from a saturated liquid culture are lysed using the detergent SDS. Proteins are removed by digestion with the nonspecific serine protease, proteinase K. Cell wall debris, polysaccharides, and remaining proteins are removed by selective precipitation with CTAB, and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Materials

TE buffer (*APPENDIX 2*)
10% sodium dodecyl sulfate (SDS)
20 mg/ml proteinase K (stored at −20°C)
5 M NaCl
CTAB/NaCl solution
24:1 chloroform/isoamyl alcohol
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)
Isopropanol
70% ethanol

Growth and lysis of bacterial cells

1. Inoculate a 5-ml liquid culture with the bacterial strain of interest. Grow in conditions appropriate for that strain (i.e., appropriate medium, drug selection, temperature) until the culture is saturated. This may take several hours to several days, depending on the growth rate.

2. Spin 1.5 ml of the culture in a microcentrifuge for 2 min, or until a compact pellet forms. Discard the supernatant.

Extraction and precipitation of DNA

3. Resuspend pellet in 567 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K to give a final concentration of 100 µg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 1 hr at 37°C.

   The solution should become viscous as the detergent lyses the bacterial cell walls. There should be no need to predigest the bacterial cell walls with lysozyme.

4. Add 100 µl of 5 M NaCl and mix thoroughly.

   This step is very important since a CTAB–nucleic acid precipitate will form if the salt concentration drops below about 0.5 M at room temperature (Murray and Thompson, 1980). The aim here is to remove cell wall debris, denatured protein, and polysaccharides complexed to CTAB, while retaining the nucleic acids in solution.

5. Add 80 µl of CTAB/NaCl solution. Mix thoroughly and incubate 10 min at 65°C.

6. Add an approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol, mix thoroughly, and spin 4 to 5 min in a microcentrifuge.

   This extraction removes CTAB–protein/polysaccharide complexes. A white interface should be visible after centrifugation.

7. Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly, and spin in a microcentrifuge for 5 min.

8. Transfer the supernatant to a fresh tube. Add 0.6 vol isopropanol to precipitate the nucleic acids (there is no need to add salt since the NaCl concentration is already

high). Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible. At this point it is possible to transfer the pellet to a fresh tube containing 70% ethanol by hooking it onto the end of a micropipet that has been heat-sealed and bent in a Bunsen flame. Alternatively, the precipitate can be pelleted by spinning briefly at room temperature.

If no stringy DNA precipitate forms in the above step, this implies that the DNA has sheared into relatively low-molecular-weight pieces. If this is acceptable, i.e., if DNA is to be digested to completion with restriction endonucleases for Southern blot analysis, chromosomal DNA can often still be recovered simply by pelleting the precipitate in a microcentrifuge.

9. Wash the DNA with 70% ethanol to remove residual CTAB and respin 5 min at room temperature to repellet it. Carefully remove the supernatant and briefly dry the pellet in a lyophilizer.

10. Redissolve the pellet in 100 µl TE buffer.

This may take some time (up to 1 hr) since the DNA is of high molecular weight. 15 µl of this DNA will typically digest to completion with 10 U EcoRI in 1 hr, which is sufficient to be clearly visible on an agarose gel, or to give a good signal during a Southern hybridization.

SUPPORT PROTOCOL

REMOVAL OF POLYSACCHARIDES FROM EXISTING GENOMIC DNA PREPS

Steps 4 through 10 of the basic protocol can be adapted for removing polysaccharides and other contaminating macromolecules from existing bacterial chromosomal DNA preparations. Simply adjust the NaCl concentration of the DNA solution to 0.7 M and proceed as described, scaling volumes of solutions up or down as appropriate so that the final concentration of CTAB is 1%. If a white interface is observed after the chloroform/isoamyl extraction, this indicates that contaminating macromolecules have been removed. The CTAB extraction step (steps 5 and 6) can be repeated several times until no interface is visible.

SHORT PROTOCOL

MINIPREP OF BACTERIAL GENOMIC DNA

1. Grow bacterial strain to saturation.
2. Spin 1.5 ml for 2 min in microcentrifuge.
3. Resuspend in 567 µl TE buffer, 30 µl of 10% SDS, and 3 µl of 20 mg/ml proteinase K. Mix and incubate 1 hr at 37°C.
4. Add 100 µl of 5 M NaCl. Mix thoroughly.
5. Add 80 µl CTAB/NaCl solution. Mix. Incubate 10 min at 65°C.
6. Extract with an equal volume of chloroform/isoamyl alcohol. Spin 5 min in microcentrifuge.
7. Transfer aqueous phase to a fresh tube. Extract with phenol/chloroform/isoamyl alcohol. Spin 5 min in microcentrifuge.
8. Transfer aqueous phase to a fresh tube. Precipitate DNA with 0.6 vol isopropanol. Wash precipitate with 70% ethanol. Remove supernatant and briefly dry pellet in lyophilizer.
9. Resuspend pellet in 100 µl TE buffer.

AITERNATE PROTOCOL

LARGE-SCALE CsCl PREP OF BACTERIAL GENOMIC DNA

This procedure is essentially a scale-up of the chromosomal miniprep described in the basic protocol, followed by additional purification on a cesium chloride gradient. This procedure may be used if large amounts of exceptionally clean genomic DNA are required, e.g., for the construction of genomic libraries.

Additional Materials

Cesium chloride
10 mg/ml ethidium bromide
CsCl-saturated isopropanol or H_2O-saturated butanol
3 M sodium acetate, pH 5.2

Beckman JA-20 rotor or equivalent
50-ml Oak Ridge centrifuge tubes
Wide-bored pipet
4-ml sealable centrifuge tubes
Beckman VTi80 rotor
3-ml plastic syringe with 15-G needle

Preparation and lysis of cells

1. Grow 100 ml culture of bacterial strain to saturation.
2. Pellet cells for 10 min at 4000 × *g* (e.g., in a Beckman JA-20 rotor at 6000 rpm). Discard supernatant.

   This, and the following steps, can be conveniently carried out using 50-ml Oak Ridge centrifuge tubes.

3. Resuspend cells gently in 9.5 ml TE buffer. Add 0.5 ml of 10% SDS and 50 µl of 20 mg/ml proteinase K. Mix thoroughly and incubate 1 hr at 37°C.

Precipitation and purification of DNA

4. Add 1.8 ml of 5 M NaCl and mix thoroughly.
5. Add 1.5 ml CTAB/NaCl solution. Mix thoroughly and incubate 20 min at 65°C.
6. Add an equal volume of chloroform/isoamyl alcohol. Extract thoroughly. Spin 10 min at 6000 × *g* (JA-20 rotor at 7000 rpm), room temperature, to separate phases.
7. Transfer aqueous supernatant to a fresh tube using a wide-bored pipet.

   The supernatant will probably be very viscous if the yield is high. An additional chloroform/isoamyl alcohol extraction, or a phenol/chloroform/isoamyl alcohol extraction, is optional but should not be necessary if the material is to be purified on a cesium chloride gradient.

8. Add 0.6 vol isopropanol and mix until a stringy white DNA pellet precipitates out of solution and condenses into a tight mass. Transfer the precipitate to 1 ml of 70% ethanol in a fresh tube, by hooking it on the end of a Pasteur pipet that has been bent and sealed in a Bunsen flame.
9. Spin the pellet 5 min at 10,000 × *g* (JA-20 rotor at 9000 rpm). Remove supernatant and redissolve the pellet in 4 ml TE buffer. This may take several hours to overnight—the DNA can be placed at 60°C to hasten the process.
10. Measure the DNA concentration on a spectrophotometer. Adjust concentration to 50 to 100 µg/ml. This will give 200 to 400 µg chromosomal DNA per 4 ml gradient.

    It is not advisable to spin larger quantities of chromosomal DNA on such a small gradient.

11. Add 4.3 g CsCl per 4 ml TE buffer. Dissolve. Add 200 µl of 10 mg/ml ethidium bromide. Transfer to 4-ml sealable centrifuge tubes. Adjust volume and balance tubes with CsCl in TE buffer (1.05 g/ml). Seal tubes. Spin 4 hr in a Beckman VTi80 rotor at 70,000 rpm, 15°C, or overnight at 55,000 rpm, 15°C.

12. Visualize gradient under longwave UV lamp. A single band should be visible. Remove band using a 15-G needle and a 3-ml plastic syringe.

    If the DNA is intact high-molecular-weight chromosomal DNA it will appear very viscous as the band is withdrawn from the gradient; hence, it is important to use a wide-bore needle to avoid mechanical shearing of the DNA. If the band appears right at the top of the gradient, then the gradient is too dense. Reduce the amount of CsCl added in step 11.

13. Remove the ethidum bromide by sequential extractions with CsCl-saturated isopropanol or water-saturated butanol, as described in UNIT 1.8.

14. Dialyze overnight against 2 liters TE buffer to remove CsCl.

15. Transfer DNA solution to a fresh tube. If required, precipitate chromosomal DNA as described above (steps 8 and 9) by adding 1/10 vol of 3 M sodium acetate and 0.6 vol isopropanol, and resuspend at desired concentration.

LARGE-SCALE CsCl PREP OF BACTERIAL GENOMIC DNA

SHORT PROTOCOL

1. Grow 100 ml culture of bacterial strain to saturation.
2. Spin 10 min at 4000 × *g*.
3. Resuspend pellet in 9.5 ml TE buffer, 0.5 ml of 10% SDS, and 50 µl of 20 mg/ml proteinase K. Mix and incubate 1 hr at 37°C.
4. Add 1.8 ml of 5 M NaCl. Mix thoroughly.
5. Add 1.5 ml CTAB/NaCl solution and mix. Incubate 20 min at 65°C.
6. Extract with an equal volume of chloroform/isoamyl alcohol. Spin 10 min at 6000 × *g*, room temperature.
7. Transfer aqueous phase to a fresh tube. Extract with phenol/chloroform/isoamyl alcohol if necessary. Spin as in step 6.
8. Transfer aqueous phase to a fresh tube. Precipitate DNA with 0.6 vol isopropanol. Wash precipitate with 70% ethanol. Remove supernatant and resuspend pellet in 4 ml TE buffer.
9. Measure DNA concentration. Adjust concentration to give 50 to 100 µg/ml. Add 4.3 g CsCl per 4 ml TE buffer. Add 200 µl of 10 mg/ml ethidium bromide. Transfer to sealable centrifuge tubes. Spin 4 hr at 70,000 rpm, 15°C.
10. Visualize gradient with UV light. Remove band.
11. Extract ethidium bromide with CsCl-saturated isopropanol.
12. Dialyze overnight against 2 liters TE buffer.

REAGENTS AND SOLUTIONS

CTAB/NaCl solution (10% CTAB in 0.7 M NaCl)

Dissolve 4.1 g NaCl in 80 ml water and slowly add 10 g CTAB (hexadecyltrimethyl ammonium bromide) while heating and stirring. If necessary, heat to 65°C to dissolve. Adjust final volume to 100 ml.

COMMENTARY

Background Information

Most commonly used protocols for the preparation of bacterial genomic DNA consist of lysozyme/detergent lysis, followed by incubation with a nonspecific protease, and a series of phenol/chloroform/isoamyl alcohol extractions prior to alcohol precipitation of the nucleic acids (Meade et al., 1984; Silhavy et al., 1982). Such procedures effectively remove contaminating proteins, but are not effective in removing the copious amounts of exopolysaccharides that are produced by many bacterial genera, and which can interfere with the activity of molecular biological enzymes such as restriction endonucleases and ligases. In this procedure, however, the protease incubation is followed by a CTAB extraction whereby CTAB complexes both with polysaccharides and with residual protein; both groups of contaminating molecules are effectively removed in the subsequent emulsification and extraction with chloroform/isoamyl alcohol. This procedure is effective in producing digestible chromosomal DNA from a variety of gram-negative bacteria, including those of the genera *Pseudomonas*, *Agrobacterium*, *Rhizobium*, and *Bradyrhizobium*, all of which normally produce large amounts of polysaccharides. If large amounts of exceptionally clean DNA are required, the procedure can be scaled up and the DNA purified on a cesium chloride gradient, as described in the alternate protocol. The method can also be used to extract high-molecular-weight DNA from plant tissue (Murray and Thompson, 1980).

Critical Parameters

The most critical parameter is the salt (NaCl) concentration of the solution containing the lysed bacteria prior to adding CTAB. If the NaCl concentration is <0.5 M then the nucleic acid may also precipitate; indeed, CTAB is frequently used for just this process (Murray and Thompson).

It is also important to maintain all solutions above 15°C, as the CTAB will precipitate below this temperature.

Anticipated Results

The typical yield from both the miniprep and the large-scale prep is 0.5 to 2 mg DNA per 100 ml starting culture (10^8 to 10^9 cells/ml).

Time Considerations

The miniprep takes ~2 hr, including the 1-hr incubation. The large-scale prep takes slightly longer to reach the point where the CsCl gradient is loaded. Subsequent steps will spread over 1 to 2 days, depending on the time of the CsCl gradient spin.

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PREPARATION AND ANALYSIS OF RNA

4

continued

Contents, continued from p. 4.0.1

INTRODUCTION

The ability to isolate clean, intact RNA has important uses in cloning genes and is essential to analyzing gene expression. RNA from any cell can be copied into double-stranded DNA and cloned, resulting in the production of a cDNA library specific to the cell type (see Chapter 6). In order for such clones to be optimally useful, it is critical that full-length RNA is used as the starting material. This chapter starts by describing several methods commonly used to isolate RNA.

The difficulty in RNA isolation is that most ribonucleases are very stable and active enzymes that require no cofactors to function. The first step in all RNA isolation protocols therefore involves lysing the cell in a chemical environment that results in denaturation of ribonuclease. The RNA is then fractionated from the other cellular macromolecules. The cell type from which RNA is to be isolated and the eventual use of that RNA will determine which of the three procedures described will be appropriate.

One of the primary uses of RNA isolation procedures is the analysis of gene expression. In order to elucidate the regulatory properties of a gene, it is necessary to know the structure and amount of the RNA produced from that gene. The second part of this chapter is devoted to techniques that are used to analyze RNA. Procedures such as S1 nuclease analysis and ribonuclease protection can be used to do fine-structure mapping of any RNA. These techniques allow characterization of 5′ and 3′ splice junctions as well as the 5′ and 3′ ends of RNA. Both of these procedures, as well as northern analysis, can also be used to accurately determine the steady-state level of any particular message.

After determining the steady-state level of a message, many investigators wish to examine whether that level is set by the rate of transcription of the gene. Alterations in steady-state level might also reflect changes in processing or stability of the RNA. The final section of the chapter describes the "nuclear run-off" technique, which determines the number of active RNA polymerase molecules that are traversing any particular segment of DNA. This procedure is used to analyze directly how the rate of transcription of a gene varies when the growth state of a cell is changed.

PREPARATION OF RNA FROM EUKARYOTIC AND PROKARYOTIC CELLS

Three methods are presented for preparing RNA from eukaryotic cells. The first two are rapid and can be used to prepare several RNA samples at the same time. As written, they are intended for production of RNA to be used for analysis by S1 nuclease or ribonuclease protection. Modifications of each protocol are given that should be used if intact, full-length RNA is a priority.

Both of these protocols require limited hands-on time. The first (p. 4.1.2) utilizes a gentle detergent to lyse the cell. Its main advantage is that it requires no high-speed centrifuge spins, thus allowing preparation of numerous samples without having to find several high-speed rotors. In the second protocol (*UNIT 4.2*) cells are lysed using 4 M guanidinium isothiocyanate. This protocol requires very few manipulations, gives clean RNA from many sources, and is the method of choice when working with tissues that have high levels of endogenous RNase. It does require a high-speed centrifuge run, which limits the number of samples that can be prepared at the same time.

In the third protocol (*UNIT 4.3*) the cell is lysed with phenol and SDS. It produces clean, full-length RNA from large quantities of plant cells. This protocol also works well with several mammalian cells and tissues. All three protocols can be used with cells from any higher eukaryote. In particular, many laboratories use the guanidinium procedure when preparing RNA from plant tissue..

These protocols produce total RNA, which contains primarily ribosomal RNA and transfer RNA. Many techniques require messenger RNA that is largely free of contaminating rRNA and tRNA. The isolation of poly(A)$^+$ RNA, which is highly enriched for mRNA, is described in *UNIT 4.5*. A protocol is also presented for extracting RNA from gram-negative and gram-positive bacteria (*UNIT 4.4*).

The major source of failure in any attempt to produce RNA is contamination by ribonuclease. RNases are very stable enzymes and generally require no cofactors to function. Therefore, a small amount of RNase in an RNA preparation will create a real problem. To avoid contamination problems, the following precautions can be taken:

1. Solutions. Any water or salt solutions used in RNA preparation should be treated with the chemical diethylpyrocarbonate (DEPC). This chemical inactivates ribonucleases by covalent modification. Solutions containing Tris cannot be effectively treated with DEPC because Tris reacts with DEPC to inactivate it. See reagents and solutions (p. 4.1.5) for instructions for DEPC treatment.
2. Glassware and plastic. Labware used in the preparation of RNA should be treated to remove residual RNase activity. Autoclaving will not fully inactivate many RNases. Glassware can be baked at 300°C for 4 hr. Plasticware can be rinsed with chloroform to inactivate RNase. Plasticware straight out of the package is generally free from contamination and can be used as is.
3. Hands are a major source of contaminating RNase. Wear gloves.

Preparation of Cytoplasmic RNA From Tissue Culture Cells

BASIC PROTOCOL

Cells are washed with ice-cold phosphate-buffered saline and kept on ice for all subsequent manipulations. The pellet of harvested cells is resuspended in a lysis buffer containing the nonionic detergent Nonidet P-40. Lysis of the plasma membranes occurs almost immediately. The intact nuclei are removed by a brief microcentrifuge spin, and sodium dodecyl sulfate (SDS) is added to the cytoplasmic supernate to denature protein. Protein is digested with protease and removed by extractions with phenol/chloroform and chloroform. The cytoplasmic RNA is recovered by ethanol precipitation and quantitated by measuring its absorbance at 260 and 280 nm.

Materials

Diethylpyrocarbonate (DEPC)
Ice-cold phosphate-buffered saline (PBS; *APPENDIX 2*)
Ice-cold lysis buffer
20% sodium dodecyl sulfate (SDS)
20 mg/ml proteinase K
25:24:1 phenol/chloroform/isoamyl alcohol
24:1 chloroform/isoamyl alcohol
3 M sodium acetate, pH 5.2
Ethanol
75% ethanol/25% 0.1 M sodium acetate, pH 5.2

Beckman J-6M rotor
Rubber policeman

Water and sodium acetate should be treated with DEPC to inhibit RNase activity. See reagents and solutions (p. 4.1.5) for instructions. CAUTION: *DEPC is a suspected carcinogen and should be handled carefully.*

1. Wash cells free of medium with ice-cold PBS. For monolayer cultures, rinse three times. For suspension cultures, pellet by centrifugation 5 min at 1000 rpm in a Beckman J-6M, resuspend in one-half the original culture volume PBS, and pellet again.

   This procedure, as written, is used for up to 4×10^7 cells (two 10-cm dishes or ~20 ml of suspension culture). The procedure can be scaled up for larger cell quantities by increasing volumes appropriately and using larger, conical tubes.

2. For monolayer cultures, scrape into a small volume of cold PBS with a rubber policeman. Transfer to a centrifuge tube on ice. Collect cells by centrifuging 5 min at 1000 rpm in a Beckman J-6M or 15 sec in a microcentrifuge. Keep cells cold.

   With a 10-cm dish, collect cells in 1 ml. With a 15-cm dish, collect in 3 to 5 ml.

3. Resuspend cells in 375 µl ice-cold lysis buffer. Incubate 5 min on ice. The suspension should clear rapidly, indicating cell lysis.

   Cells are best suspended by careful but vigorous vortexing. Avoid foaming.

4. If the cells are not already in a microcentrifuge tube, transfer them into one. Spin 2 min at 4°C.

5. Remove the supernatant fluid to a clean tube containing 4 µl of 20% SDS. Mix immediately by vortexing.

   The supernatant fluid is the cytoplasmic extract. It is usually slightly cloudy and yellow-white, depending on the cells. The pellet, which contains nuclei and some

cell debris, should be considerably smaller than the whole cell pellet obtained in step 2 and white in color.

6. Add 2.5 µl of 20 mg/ml proteinase K. Incubate 15 min at 37°C.

7. Add 400 µl phenol/chloroform/isoamyl alcohol. Vortex thoroughly—at least 1 min. Spin for ≥ 5 min in a microcentrifuge. The extractions and centrifugations are performed at room temperature.

   With protease treatment, there should be only a small amount of precipitate at the interface between the two phases, although this can vary depending on the cell type. For some cells, the protease step can be safely omitted. In this case, the white precipitate at the interface can be considerable. If a very large precipitate forms after the first organic extraction and little or no aqueous phase can be recovered, first try spinning for a few minutes more. If the precipitate fails to collapse to the interface, remove and discard the organic phase from the bottom of the tube. Add 400 µl chloroform/isoamyl alcohol. Vortex well and spin ~2 min. The precipitate should have largely disappeared. Recover the upper aqueous phase and proceed.

8. Remove the aqueous (upper) phase to a clean tube, avoiding precipitated material from the interface. Add 400 µl phenol/chloroform/isoamyl alcohol and repeat the extraction.

9. Remove the aqueous phase to a clean tube. Add 400 µl chloroform/isoamyl alcohol. Vortex 15 to 30 sec and spin 1 min.

10. Again, remove the aqueous (upper) phase to a clean tube.

11. Add 40 µl of 3 M sodium acetate, pH 5.2, and 1 ml ethanol. Mix by inversion. Incubate 15 to 30 min on ice or store at –20°C overnight.

12. Recover the RNA by centrifugation for 15 min at 4°C.

13. Rinse the pellet with 1 ml of 75% ethanol/25% 0.1 M sodium acetate, pH 5.2 solution.

14. Dry and resuspend in 100 µl DEPC-treated water. Dilute 10 µl into 1 ml water to determine the A_{260} and A_{280}. Store the remaining RNA at –70°C.

SHORT PROTOCOL

PREPARATION OF CYTOPLASMIC RNA FROM TISSUE CULTURE CELLS

1. Wash cells with ice-cold PBS. Collect by centrifugation (5 min, 1000 rpm).
2. Resuspend in 375 µl ice-cold lysis buffer. Incubate on ice 5 min.
3. Transfer to microcentrifuge tube. Spin 2 min at 4°C.
4. Remove supernatant fluid to clean tube containing 4 µl of 20% SDS. Mix.
5. Add 2.5 µl of 20 mg/ml proteinase K. Incubate 15 min at 37°C.
6. Extract with 400 µl phenol/chloroform/isoamyl alcohol. Recover the aqueous (upper) phase.
7. Repeat phenol/chloroform/isoamyl alcohol extraction.
8. Extract with 400 µl chloroform/isoamyl alcohol. Recover the aqueous phase.
9. Add 40 µl of 3 M sodium acetate, pH 5.2, and 1 ml ethanol. Precipitate 15 to 30 min on ice or overnight at –20°C.
10. Collect RNA by centrifugation for 15 min at 4°C.
11. Rinse pellet with 1 ml of 75% ethanol/25% 0.1 M sodium acetate, pH 5.2.
12. Dry. Redissolve in 100 µl water. Dilute 10 µl in 1 ml water to determine the A_{260} and A_{280}.
13. Store RNA at –70°C.

SUPPORT PROTOCOL

REMOVAL OF CONTAMINATING DNA

If RNA is isolated from cells transiently transfected with cloned DNA, substantial amounts of this DNA will copurify with the RNA in this procedure. This contaminating DNA will interfere with analysis of the RNA by nuclease protection assays, especially if uniformly labeled probes are used. To remove this DNA, perform the following steps after step 12 of the basic protocol.

Additional Materials

TE buffer, pH 7.4 (*APPENDIX 2*)
100 mM $MgCl_2$/10 mM dithiothreitol (DTT) solution
2.5 mg/ml RNase-free DNase I
Placental ribonuclease inhibitor (e.g., RNAsin from Promega Biotec)
  or vanadyl-ribonucleoside complex
DNase stop mix

1. Redissolve the RNA in 50 µl TE buffer.
2. Prepare on ice a cocktail containing (per sample) 10 µl of 100 mM $MgCl_2$/10 mM DTT solution, 0.2 µl of 2.5 mg/ml RNase-free DNase, 0.1 µl placental ribonuclease inhibitor (25 to 50 U/µl), and 39.7 µl TE buffer. Add 50 µl of this cocktail to each RNA sample. Mix and incubate 15 min at 37°C.
3. Stop the DNase reaction by adding 25 µl DNase stop mix.
4. Extract once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol.

5. Add 325 µl ethanol and precipitate 15 to 30 min on ice or overnight at −20°C.

Resume basic protocol at step 12.

REAGENTS AND SOLUTIONS

Diethylpyrocarbonate (DEPC) treatment of solutions

Add 0.2 ml DEPC to 100 ml of the solution to be treated. Shake vigorously to get the DEPC into solution. Autoclave the solution to inactivate the remaining DEPC. Many investigators keep the solutions they use for RNA work separate to ensure that "dirty" pipets do not go into them.

CAUTION: *Wear gloves and use a fume hood when using DEPC, as it is a suspected carcinogen.*

DNase stop mix

50 mM EDTA
1.5 M sodium acetate
1% sodium dodecyl sulfate (SDS)

The SDS may come out of solution at room temperature. Heat briefly to redissolve.

Lysis buffer

50 mM Tris·Cl, pH 8.0
100 mM NaCl
5 mM $MgCl_2$
0.5% (vol/vol) Nonidet P-40
Prepare with DEPC-treated H_2O (see above)
Filter sterilize

If the RNA is to be used for northern blot analysis or the cells are particularly rich in ribonuclease, add ribonuclease inhibitors to the lysis buffer: 1000 U/ml placental ribonuclease inhibitor (e.g., RNAsin) plus 1 mM DTT or 10 mM vanadyl-ribonucleoside complex.

RNase-free DNase I

Commercially prepared enzymes such as Worthington grade DPRF (#LS0633) are satisfactory. If supplied as a powder, redissolve in TE buffer containing 50% (vol/vol) glycerol and store at −20°C. See also UNIT 4.10, reagents and solutions, for homemade preparation of RNase-free DNase.

COMMENTARY

Background Information and Literature Review

Most procedures for isolating RNA from eukaryotic cells involve lysing and denaturing cells to liberate total nucleic acids. Additional steps are then required to remove DNA. This procedure allows rapid preparation of total cytoplasmic RNA by using a nonionic detergent to lyse the plasma membrane, leaving the nuclei intact. The nuclei and hence the bulk of the cellular DNA are then removed with a simple brief centrifugation.

Variations of this technique are in wide use, and its precise origins are obscure. Versions of this procedure were used in some of the early S1 nuclease mapping papers (Berk and Sharp, 1977; Favoloro et al., 1980). The protocol described here is a considerable simplification of the earlier methods, omitting, for example, removal of nuclei by centrifugation through sucrose. It is fast and streamlined, designed for preparing total cytoplasmic RNA from many cultures simultaneously for nuclease protection analysis. It is scaled for small cultures—1 to 2 dishes of adherent cells or 10 to 20 ml of a suspension culture.

The basic protocol works well for many cell types. The protocol takes no special precautions for ribonucleases and may not yield northern blot-quality RNA from some cells. If full-length RNA is required, ribonuclease inhibitors should be added to the lysis buffer

(as described in reagents and solutions) or the guanidinium isothiocyanate method should be used (*UNIT 4.2*). Finally, if RNA is isolated from transiently transfected cells, the RNA should be further treated with deoxyribonuclease to remove transfected DNA (see support protocol). This modification is especially critical if the RNA is to be assayed by nuclease protection using uniformly labeled probes.

Troubleshooting

Degradation of RNA by ribonuclease is best avoided by working quickly and keeping everything cold until SDS is added to the cytoplasmic extract. For cells with which ribonuclease is a problem, inhibitors can be added to the lysis buffer (see reagents and solutions), but in most cases this is unnecessary.

Note that for some cell lines, it may be possible to omit the proteinase K step and to proceed directly to organic extraction after removal of the nuclei and addition of SDS.

DNA contamination is only a problem when preparing RNA from cells transfected with cloned DNA in a transient expression assay. In this case, add the DNase digestion steps outlined in the support protocol.

Anticipated Results

Yields vary widely, depending on the cell line. Expect 30 to 100 μg from a confluent 10-cm dish of most fibroblast lines or 10^7 lymphoid cells. Ratios of A_{260} to A_{280} should fall in the range 1.7 to 2.0. RNA at 1 mg/ml has an A_{260} of 25.

Time Considerations

Depending on the number of samples being processed, it is possible to proceed from harvesting the cells to the ethanol precipitation step in 1 to 2 hr. This is the best interim stopping point. The RNA may be recovered, redissolved, and quantitated later the same day or the following day.

Literature Cited

Berk, A. J. and Sharp, P. A. 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* 12: 721-732.

Favoloro, J., Treisman, R., and Kamen, R. 1980. Transcription maps of polyoma virus-specific RNA: Analysis by two-dimensional nuclease S1 gel mapping. *Meth. Enzymol.* 65:718-749.

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Guanidinium Method for Total RNA Preparation

BASIC PROTOCOL

PREPARATION OF RNA FROM CULTURED CELLS

Cells are washed free of media and are then lysed by placing them in a 4 M guanidinium solution. The viscosity of the solution is reduced by drawing the lysate through a 20-G needle. The RNA is then pelleted through a CsCl step gradient. The supernatant fluid from this gradient is then carefully removed to allow complete separation of RNA, found in the pellet, from contaminating DNA and protein.

Materials

Diethylpyrocarbonate (DEPC)
Phosphate-buffered saline (PBS; *APPENDIX 2*)
Guanidinium solution
5.7 M CsCl
TES solution
3 M sodium acetate
Ethanol

Beckman J-6M rotor or equivalent
Policeman
6-ml syringe with 20-G needle
Siliconized and autoclaved 13 × 51 mm polyallomer ultracentrifuge tube (*APPENDIX 3*)
Beckman SW-55 rotor or equivalent

The following solutions should be treated with DEPC to inhibit RNase activity: 5.7 M CsCl, sodium acetate, and water. See *UNIT 4.1*, reagents and solutions, for instructions. CAUTION: *DEPC is a suspected carcinogen and should be handled carefully.*

Lysis of Cells

1. Wash cells with PBS. For monolayer cells, wash twice, each time with 5 ml. For suspension culture, pellet cells by centrifuging 5 min at 1000 rpm in a Beckman J-6M rotor (or equivalent), resuspend the cells in an amount of PBS equal to one-half the original volume, and pellet again.

   Steps 1 to 4 are done at room temperature.

2. Add 3.5 ml guanidinium solution to cells. Cells should immediately lyse. The 3.5 ml guanidinium solution is sufficient for up to 10^8 cells.

   If RNA is being isolated from monolayer culture, this step is most easily done by dividing the 3.5 ml equally onto dishes, thus lysing the cells in place. Recover the viscous lysate by scraping the dishes with a policeman. Remove lysate from dish using a 20-G needle fitted on a 6-ml syringe.

3. Draw the resultant extremely viscous solution up and down four times through a 20-G needle. Transfer the solution to a clean tube.

   It is critical that chromosomal DNA is sheared in this step in order to reduce viscosity. This allows complete removal of the DNA in the centrifugation step.

Isolation of RNA

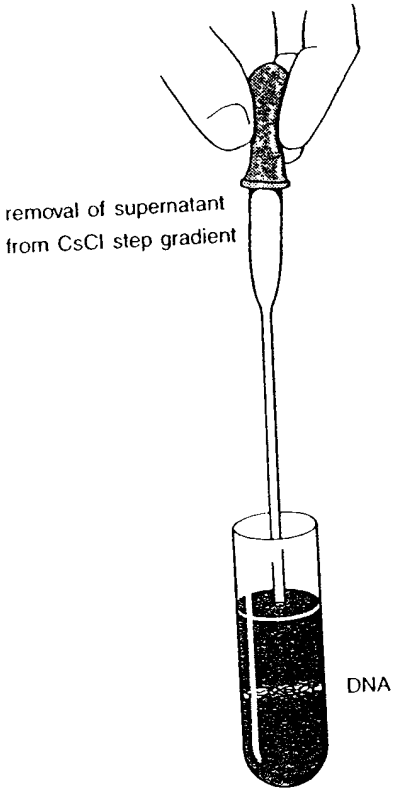
4. Place 1.5 ml of 5.7 M CsCl in a 13 × 51 mm siliconized and autoclaved polyallomer ultracentrifuge tube. Layer 3.5 ml of cell lysate on top of CsCl cushion to create a step gradient. The interface should be visible.

   Siliconizing the tube decreases the amount of material that sticks to the sides of the tube and thus decreases the level of contamination of the final RNA.

5. Centrifuge at 35,000 rpm in a Beckman SW-55 rotor (or equivalent) 12 to 20 hr at 18°C. Set centrifuge for slow acceleration and deceleration in order to avoid disturbing the gradient.

6. Remove the supernatant very carefully (see sketch). Place the end of the Pasteur pipet at the top of the solution and lower it as the level of the solution lowers. Leave roughly 100 µl in the bottom, invert the tube carefully, and pour the remaining liquid off.

   There should be a white band of DNA at the interface—care must be taken to remove this band completely, as it contains cellular DNA.



7. Drain the pellet 5 to 10 min, then resuspend it in 360 µl TES solution. Allow the pellet to resuspend 5 to 10 min at room temperature, and transfer to a clean microcentrifuge tube.

   It is critical to allow ample time for resuspension of this pellet or yield of RNA will be significantly decreased.

8. Add 40 µl of 3 M sodium acetate and 1 ml ethanol, and precipitate the RNA 30 min on dry ice/ethanol.

9. Resuspend the pellet in 360 µl water and reprecipitate with 3 M sodium acetate and ethanol as above.

   RNA dissolves more readily in water than in a salt solution.

10. Dissolve the pellet in water and quantitate by diluting 10 µl to 1 ml and reading the A_{260} and A_{280}. Store RNA at –70°C either as an aqueous solution or as an ethanol precipitate.

    This protocol produces RNA that is clean enough for Northern, S1, or SP6 analysis. If cleaner RNA is desired, step 7 can be substituted with the following: Extract with

360 µl chloroform/1-butanol (4:1, vol/vol), and save the supernatant. Extract the chloroform by adding 360 µl TES solution. Combine the supernatants, add 0.1 vol of 3 M sodium acetate, and ethanol precipitate.

ALTERNATE PROTOCOL

EXTRACTION OF RNA FROM TISSUE

There are many more contaminants and debris to remove when purifying RNA from tissue compared with tissue culture cells. This protocol is essentially as described in Chirgwin et al. (1979) and modified by Richard Selden.

Additional Materials

Liquid nitrogen
Tissue guanidinium solution
20% *N*-lauryl sarcosine (Sarkosyl)
Tissue resuspension buffer
25:24:1 phenol/chloroform/isoamyl alcohol
24:1 chloroform/isoamyl alcohol

Tissuemizer
Beckman JA-13 rotor or equivalent
Beckman SW-28 rotor or equivalent
Siliconized SW-28 polyallomer tube (*APPENDIX 3*)

1. After removing tissue from the animal, quick-freeze it in liquid nitrogen. The sample should be in pieces ≤2 g or it will be difficult to do the further workup.

   RNA is very unstable in tissue once removed from the body, so it is critical to quick freeze the tissue. Placing the tissue in guanidinium and then waiting to grind it will result in degraded RNA. Pancreas and spleen have very high endogenous levels of RNase, while liver and intestine are relatively low in RNase.

2. For ~2 g of tissue, add 20 ml tissue guanidinium solution (does not contain Sarkosyl) and grind the tissue in a tissuemizer. Two or three 10-sec bursts are required for complete grinding.

   It is important that Sarkosyl not be present, or a frothy mess will result.

3. Spin 10 min at 10,000 rpm, 12°C, in a Beckman JA-13 rotor (or equivalent).

4. Collect the supernatant fluid and add 1/10 vol 20% Sarkosyl. Heat at 65°C for 2 min.

5. Add 0.1 g CsCl/ml of solution, dissolve the CsCl, then layer the sample over 9 ml of 5.7 M CsCl in a siliconized SW-28 polyallomer tube. Spin overnight at 25,000 rpm in a Beckman SW-28 rotor (or equivalent).

6. Carefully remove the supernatant as described and illustrated in step 6 of the basic protocol. Invert the tube to drain. Cut off bottom of tube (containing RNA pellet) and place it in a 50-ml plastic tube.

7. Add 3 ml tissue resuspension buffer and allow pellet to resuspend at 4°C overnight.

   It is difficult to resuspend this pellet. Occasionally the sample may have to be left longer than overnight. The high concentrations of β-mercaptoethanol and Sarkosyl prevent RNA degradation during this resuspension.

8. Extract the solution with phenol/chloroform/isoamyl alcohol, then with chloroform/isoamyl alcohol.

9. Add 1/10 vol of 3 M sodium acetate and 2.5 vol ethanol, precipitate, and resuspend the RNA in water. Quantitate and store as described in step 10 of the basic protocol.

REAGENTS AND SOLUTIONS

5.7 M CsCl, DEPC-treated

Dissolve CsCl in 0.1 M EDTA. Add 0.002 vol DEPC, shake 20 to 30 min, and autoclave. Weigh the bottle of solution before and after autoclaving and make up the weight lost to evaporation during autoclaving with DEPC-treated H_2O. This ensures that the solution is actually 5.7 M when used.

Guanidinium solution

4 M guanidinium isothiocyanate
20 mM sodium acetate, pH 5.2
0.1 mM dithiothreitol (DTT)
0.5% *N*-lauryl sarcosine (Sarkosyl)

Dissolve the guanidinium isothiocyanate in H_2O and the appropriate amount of sodium acetate. Heating the solution slightly (65°C) may be necessary to get the guanidinium into solution. Add the DTT and Sarkosyl. Check the pH—it should be ~ 5.5. If not, adjust with acetic acid. Bring to volume and filter the solution through a Nalgene filter. Store at room temperature.

TES solution

10 mM Tris·Cl, pH 7.4
5 mM EDTA
1% sodium dodecyl sulfate

Tissue guanidinium solution, 1 liter

Dissolve 590.8 g guanidinium isothiocyanate in ~400 ml DEPC-treated H_2O (see UNIT 4.1). Add 25 ml of 2 M Tris·Cl, pH 7.5 and 20 ml of 0.5 M Na_2EDTA, pH 8.0. Stir overnight, adjust the volume to 950 ml, and filter. Finally, add 50 ml β-mercaptoethanol.

Tissue resuspension buffer

5 mM EDTA
0.5% *N*-lauryl sarcosine (Sarkosyl)
5% β-mercaptoethanol

COMMENTARY

Background Information

Cells are lysed with a solution that contains 4 M guanidinium as well as a mild detergent. This lysis is virtually instantaneous, and the cells are thus rapidly taken from an intact state to a completely denaturing environment. The protocol then takes advantage of the observation that RNA can be separated from DNA and protein by virtue of its greater density. This protocol has received widespread use because it requires very few manipulations. This increases the chance of producing intact RNA and reduces hands-on time for the experimenter. The disadvantage is that it requires an ultracentrifuge and rotor, generally limiting the number of samples that can easily be done simultaneously.

Literature Review

The use of guanidinium to lyse cells was originally developed to allow purification of RNA from cells high in endogenous ribonucleases (Ullrich et al., 1977; Chirgwin et al., 1979). Guanidinium denatures protein and thus inactivates any ribonucleases that are present. The use of the CsCl step gradient to then separate the RNA from the other cellular macromolecules (Glisin et al., 1974) is based on the observation that RNA is denser than DNA or protein.

Critical Parameters

As with any RNA preparative procedure, care must be taken to ensure that solutions are free of ribonuclease. Solutions that come into contact with the RNA after the guanidinium solution are all treated with DEPC, with the exception of the TES solution (Tris inactivates DEPC). Most investigators wear gloves at all times when working with RNA solu-

tions, as hands are a likely source of ribonuclease contamination.

This protocol relies on a thorough separation of DNA and protein from RNA in the step gradient. The use of siliconized tubes, as well as careful technique when removing the supernatant, are important. Finally, low yields may result from failing to allow sufficient time for resuspension of the RNA pellet after centrifugation. This pellet is not readily soluble, and sufficient time and vortexing should be allowed to dissolve it.

Anticipated Results

Approximately 200 μg RNA should be recovered from 10^8 cells.

Time Considerations

Harvest of the RNA and setting up the gradient takes very little time (~1 hr for six samples) and is conveniently done in the evening, allowing the high-speed centrifuge run to go overnight. In a pinch, the guanidinium cell lysate can be quick frozen in dry ice/ethanol and stored at −70°C. When the RNA is dissolved after the gradient, it can be stored as an ethanol precipitate indefinitely at any of the precipitation steps. The entire protocol requires 2 to 3 hr of hands-on time for 6 to 12 samples.

Literature Cited

Chirgwin, J.J., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294.

Glisin, V., Crkvenjakov, R., and Byus, C. 1973. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* 13:2633.

Ullrich, A., Shine, J., Chirgwin,J., Pictet, R., Tischer, E., Rutter, W.J., and Goodman, H.M. 1977. Rat insulin genes: Construction of plasmids containing the coding sequences. *Science* 196:1313.

Key Reference

Chirgwin et al., 1979. See above.

Describes the use of guanidinium to lyse cells.

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Phenol/SDS Method for Plant RNA Preparation

This protocol is divided into two stages: (1) lysis of the cells and removal of proteins by phenol/SDS extraction, and (2) separation of RNA from DNA and other impurities by selective precipitation using LiCl.

Materials

Diethylpyrocarbonate (DEPC)
Liquid nitrogen
Grinding buffer
Phenol equilibrated with TLE
Chloroform
8 M LiCl
2 M LiCl
3 M sodium acetate
Ethanol

Polytron (Brinkmann PT 10/35)
Beckman JA-10 rotor
50-ml Oak Ridge tube
Beckman JA-20 rotor
Beckman JA-14 rotor
Sarstedt tube

Sodium acetate, water, and LiCl solutions should be treated with DEPC to inhibit RNase activity. See UNIT 4.1, reagents and solutions, for instructions. CAUTION: *DEPC is a suspected carcinogen and should be handled carefully.*

Tissue homogenization and protein extraction

1. Cool a mortar and pestle by pouring a little liquid nitrogen over it.
2. Weigh 15 g frozen plant tissue. If using freshly harvested tissue, quick-freeze in liquid nitrogen.

   The time between harvesting of the tissue and freezing should be minimized; once tissue is frozen, work quickly so it does not have a chance to thaw.

3. Grind plant tissue in the mortar and pestle until tissue becomes a fine powder.

   Add liquid nitrogen as needed to keep tissue frozen.

4. Immediately add powdered tissue to 150 ml grinding buffer plus 50 ml TLE-equilibrated phenol in 500-ml beaker.
5. Homogenize the mixture with Polytron for ~2 min at moderate speed (setting 5-6).
6. Add 50 ml chloroform. Use Polytron at low speed to mix in the added chloroform.

   It is not necessary to add isoamyl alcohol to the chloroform for the following extractions.

7. Pour the slurry into a 500-ml Nalgene centrifuge bottle and heat 20 min at 50°C.

   All extractions involving TLE-equilibrated phenol and chloroform should be done in screw-cap tubes or bottles resistant to those chemicals.

8. Centrifuge mixture 20 min at 10,000 rpm, 4°C, in a Beckman JA-10 rotor.
9. Take off as much aqueous layer as possible without disturbing the interface and transfer it to a clean 500-ml Nalgene bottle. Add 50 ml TLE-equilibrated phenol to this aqueous layer, shake bottle to mix phenol and aqueous phase, then add 50 ml chloroform.

TLE-equilibrated phenol and chloroform are added to the freshly removed aqueous layer to reduce possibility of degradation of RNA while steps 10 and 11 are performed.

10. Remove remaining aqueous layer together with interface from initial phenol extraction and transfer it to a 50-ml Oak Ridge tube. Centrifuge this material 20 min at 10,000 rpm, 4°C, in JA-20 rotor.

11. Remove aqueous layer and combine with aqueous phase already separated in step 9.

    Steps 10 and 11 are done to recover the large volume of aqueous phase that is difficult to separate from the interface in a 500-ml bottle.

12. Vigorously shake the 500-ml bottle containing the combined aqueous layers to mix TLE-equilibrated phenol and chloroform with aqueous phase. Centrifuge mixture 15 min at 10,000 rpm, 4°C, in JA-10 rotor and remove aqueous layer to fresh 500-ml bottle.

13. Reextract the aqueous phase with TLE-equilibrated phenol and chloroform until no interface is obtained (usually a total of three extractions).

    The interface should be small on these steps, so recentrifuging as in steps 10 and 11 is not necessary.

14. Extract aqueous phase one last time with chloroform.

    This removes traces of TLE-equilibrated phenol in the aqueous layer which can cause problems with the lithium chloride precipitation.

Selective precipitation of RNA

These steps result in removal of contaminating DNA. If there is no need to remove DNA—for example, when poly(A)$^+$ selection is the next step—simply ethanol precipitate the nucleic acid.

15. Transfer the aqueous phase to a clean 250-ml Nalgene bottle and add 8 M LiCl ($^1/_3$ vol) to bring solution to a final concentration of 2 M LiCl. Precipitate overnight at 4°C.

16. Collect precipitate by centrifugation for 20 min at 10,000 rpm, 4°C, in Beckman JA-14 rotor. Rinse pellet with a few milliliters of 2 M LiCl.

17. Resuspend pellet in 5 ml water and transfer to a 15-ml Sarstedt tube. Add 8 M LiCl to bring concentration of LiCl to 2 M and precipitate the RNA at 4°C for at least 2 hr.

18. Recover the RNA by centrifugation for 20 min at 10,000 rpm, 4°C, in JA-20 rotor. Rinse pellet with 2 M LiCl.

19. Resuspend RNA pellet in 2 ml water. Add 200 µl of 3 M sodium acetate and 5.5 ml ethanol. Precipitate at −20° overnight or in dry ice/ethanol for 30 min.

    RNA can be stored in ethanol at −20° or −70°C indefinitely.

20. Recover RNA by centrifugation for 15 min at 10,000 rpm, 4°C, in JA-20 rotor. Resuspend RNA in 1 ml water. Dilute 10 µl to 1 ml and measure the A_{260} and A_{280}. 1 OD_{260} = 40 µg/ml RNA.

REAGENTS AND SOLUTIONS

Grinding buffer

0.18 M Tris
0.09 M LiCl
4.5 mM EDTA
1% sodium dodecyl sulfate (SDS)
pH to 8.2 with HCl
This buffer is equivalent to TLE solution with 1/10 vol 10% SDS added.

Phenol

Equilibrate freshly liquefied phenol (250 ml for a 15-g prep) with TLE solution (see below) on the day of preparation. First, extract with an equal volume of TLE solution plus 0.5 ml of 15 M NaOH (this should bring the pH close to 8.0), then extract two more times with TLE solution.

TLE solution

0.2 M Tris
0.1 M LiCl
5 mM EDTA
pH to 8.2 with HCl

COMMENTARY

Background Information

The method described here can be used to prepare RNA from a variety of eukaryotic tissues. The critical factor in isolating RNA from eukaryotic tissues is inactivating the endogenous RNase and preventing introduction of RNase from external sources. In general, protocols for making RNA from eukaryotic organisms involve lysing the cells in the presence of a strong denaturant and deproteinizing agent which inhibits RNase as well as strips the protein away from the RNA. In this protocol, the RNA is then separated from DNA and other impurities by selective precipitation in high salt.

Literature Review

The use of phenol in RNA preparations originated with a method described by Kirby (1968). The phenol/SDS procedure for RNA extraction described here is taken most directly from a protocol developed by Palmiter (1974). However, this procedure has evolved from Palmiter's through contributions from many different laboratories. The most widely used alternative method, developed by Chirgwin et al. (1979), involves the use of guanidinium isothiocyanate instead of the phenol/SDS mixture to disrupt cells and inactivate nucleases.

Critical Parameters

The most essential factor in making high-quality RNA from eukaryotic tissues is to eliminate RNase activity. The endogenous RNase is quickly inactivated by the phenol/SDS extraction. After the extractions, it is very important not to introduce RNase from external sources. In making RNA from plant tissues, special attention must be paid to effectively grinding up the tissue. Plant tissues are frequently fibrous and contain organic compounds that can make fresh tissue difficult to break up. Therefore, it is recommended that the tissue is frozen before grinding in a mortar and pestle.

Anticipated Results

The yield of RNA varies widely, depending on the plant tissue from which the RNA is extracted. A tissue that is good for making RNA, such as pea seedlings, should yield about 7 mg of total RNA from 15 g of starting material; however, mature *Arabidopsis* plants yield only about 3 mg from the same amount of tissue. The quality of RNA will also vary due to differences in levels of carbohydrates and secondary metabolites in the tissues used.

This protocol can easily be scaled up or down by appropriate changes in the volumes used. It can be successfully used with less than 1 g of tissue.

Time Considerations

Two samples will take 2 to 3 hr to process to the first LiCl precipitation. After the addition of LiCl to the aqueous phase of the phenol extractions, the RNA can be stored at 4°C for days or even weeks.

Literature Cited

Chirgwin, J.M., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294.

Kirby, K.S. 1968. Isolation of nucleic acids with phenolic solvents. *Meth. Enzymol.* 12B:87.

Palmiter, R.D. 1974. Magnesium precipitation of ribonucleoprotein complexes: Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. *Biochemistry* 13:3606.

Preparation of Bacterial RNA

The basic protocol uses sonication to break open the cell wall, detergent to lyse the membranes, and protease digestion to degrade cellular protein. Organic extraction and ethanol precipitation yield total nucleic acids. DNA is removed enzymatically, and the RNA is repurified. In the alternate procedure, lysozyme is used to strip off the cell walls, and the resulting protoplasts are lysed with detergent. Diethylpyrocarbonate, a potent inactivator of ribonuclease, is added to the lysate. Salt is then added to coprecipitate the detergent, protein, and chromosomal DNA. This material is removed by centrifugation. The supernatant fluid is predominantly RNA, which is recovered by ethanol precipitation.

ISOLATION OF RNA FROM GRAM-POSITIVE BACTERIA

BASIC PROTOCOL

Materials

Diethylpyrocarbonate (DEPC)
Lysis buffer
25:24:1 phenol/chloroform/isoamyl alcohol
24:1 chloroform/isoamyl alcohol
5 M NaCl
Ethanol
DNase digestion buffer
2.5 mg/ml RNase-free DNase I (*UNIT 4.1*)
TE buffer, pH 8.0 (*APPENDIX 2*)

Sorvall SS-34 rotor or equivalent
Microtip sonicator

Water and NaCl should be treated with DEPC to inhibit RNase activity. See *UNIT 4.1*, reagents and solutions, for instructions. CAUTION: *DEPC is a suspected carcinogen and should be handled carefully.*

1. Harvest cells from a 10-ml culture by centrifuging 10 min at 10,000 rpm in a Sorvall SS-34 rotor or equivalent.
2. Resuspend cells in 0.5 ml lysis buffer. Transfer to microcentrifuge tube and freeze on dry ice.
3. Thaw and sonicate three times for 10 sec with a microtip sonicator. Use a power setting of about 30 W. The cell suspension should clear, indicating lysis.

   Avoid foaming the lysate.

4. Incubate 60 min at 37°C.

   This incubation allows digestion of bacterial protein.

5. Add an equal volume of phenol/chloroform/isoamyl alcohol, spin 5 min in a microcentrifuge. Remove aqueous (top) layer to a clean microcentrifuge tube.
6. Reextract once with an equal volume of phenol/chloroform/isoamyl alcohol, then extract once with an equal volume of chloroform/isoamyl alcohol.
7. To 400 µl aqueous phase, add 15 µl of 5 M NaCl and fill microcentrifuge tube with ethanol. Mix and incubate overnight at –20°C.
8. Spin down precipitated RNA in microcentrifuge tube 15 min at 4°C. Rinse and dry the pellet.

9. Redissolve in 95 µl DNase digestion buffer. Add 4 µl of 2.5 mg/ml RNase-free DNase I. Incubate 60 min at 37°C.
10. Extract once with phenol/chloroform/isoamyl alcohol. Add 100 µl TE buffer to remaining organic phase, mix thoroughly, and spin 5 min in microcentrifuge. Pool the two aqueous phases.
11. Extract once with chloroform/isoamyl alcohol.
12. Add 10 µl of 5 M NaCl to the aqueous phase; add 600 µl ethanol. Precipitate overnight at –20°C or 15 min on dry ice/ethanol. Collect the precipitate by spinning 15 min at 4°C.
13. Rinse and dry pellet. Redissolve in 100 µl DEPC-treated water. Dilute 10 µl into 1 ml water and determine the A_{260} and A_{280}. Store the remaining RNA at –70°C.

ALTERNATE PROTOCOL

RAPID ISOLATION OF RNA FROM GRAM-NEGATIVE BACTERIA

The following rather simple procedure works well for *Escherichia coli* and other gram-negative bacteria. RNA prepared by this method contains small amounts of DNA and protein but should be adequate for most kinds of analysis.

Additional Materials

Protoplasting buffer
50 mg/ml lysozyme
Gram⁻ lysing buffer
Saturated NaCl

1. Collect cells from a 10-ml culture by centrifuging 10 min at 10,000 rpm in a Sorvall SS-34 rotor or equivalent.
2. Resuspend in 10 ml protoplasting buffer. Add 80 µl of 50 mg/ml lysozyme. Incubate 15 min on ice.

   Lysozyme digests the cell walls, leaving behind protoplasts, in effect naked cells.
3. Collect the protoplasts by centrifuging 5 min at 7000 rpm in an SS-34 rotor.

   The gentler spin is used because protoplasts are fragile.
4. Resuspend in 0.5 ml gram⁻ lysing buffer. Add 15 µl DEPC. Mix gently and transfer to a microcentrifuge tube.

   The DEPC inactivates ribonucleases in the lysate. CAUTION: *It is a suspected carcinogen and should be handled carefully with gloves.*

   The lysate should become clear and viscous. Avoid excessive agitation which shears DNA.
5. Incubate 5 min at 37°C.
6. Chill on ice. Add 250 µl saturated NaCl. Mix by inversion. A substantial precipitate should form.

   The precipitate contains sodium dodecyl sulfate, protein, and DNA.
7. Incubate 10 min on ice. Centrifuge 10 min.
8. Remove the supernatant to two clean microcentrifuge tubes. Add to each tube 1 ml ethanol and precipitate 30 min on dry ice or overnight at –20°C.
9. Centrifuge 15 min at 4°C.

10. Rinse and dry pellet. Redissolve in 100 µl water and determine the UV absorbance (A_{260} and A_{280}) as in the basic protocol.

REAGENTS AND SOLUTIONS

DNase digestion buffer
20 mM Tris·Cl, pH 8.0
10 mM $MgCl_2$

Gram⁻ lysing buffer
10 mM Tris·Cl, pH 8.0
10 mM NaCl
1 mM Na-citrate
1.5% sodium dodecyl sulfate

Lysis buffer
30 mM Tris, pH 7.4
100 mM NaCl
5 mM EDTA
1% SDS
Add proteinase K to 100 µg/ml just before use

Protoplasting buffer
15 mM Tris·Cl, pH 8.0
0.45 M sucrose
8 mM EDTA

Saturated NaCl
Add 40 g NaCl to 100 ml water. Stir until solution reaches saturation.

COMMENTARY

Background Information

Most procedures for isolating RNA from any biological source involve disruption of the cells, followed by steps to remove DNA and protein. Several strategies have been used to achieve these ends. For example, chromosomal DNA may be removed by precipitation with SDS and salt or it may be digested enzymatically. Protein is usually removed by a combination of enzymatic digestion and organic extraction. What remains after these manipulations is predominantly RNA, which is eventually recovered and concentrated by ethanol precipitation.

Literature Review

The basic protocol is an adaptation of published methods for preparing RNA from eukaryotic cells (McKnight, 1978; Thomas, 1980) for use with the gram-positive bacterium *Bacillus subtilis* (Gilman and Chamberlin, 1983). It is a relatively straightforward technique which uses protease digestion and organic extraction to remove protein and nuclease digestion to remove DNA. The principal complication is that sonication is usually required to facilitate lysis of *B. subtilis*. This procedure may also be used for *E. coli*. The alternate protocol provides a relatively simple method for rapidly isolating RNA from *E. coli*, without organic extractions, protease, or nuclease treatment (Summers, 1970).

Critical Parameters

In the basic protocol, care should be taken to avoid foaming the lysate while sonicating. Recall that the lysate contains SDS. The critical step in the alternate protocol is lysis. Care should be exercised here to avoid manipulations which might shear the chromosomal DNA. Sheared fragments of DNA will not be efficiently removed from the lysate by the salt precipitation.

Anticipated Results

From 10 ml cells at A_{600} = 1 in rich medium, expect about 0.5 to 1 mg RNA, more than 95% of which is stable rRNA and

tRNA. Yields from cells growing more slowly in poorer media are lower, but this difference is predominantly due to the stable RNAs whose rate of synthesis is tightly coupled to growth rate.

Time Considerations

For the basic protocol, allow about 3 hr to get to the first ethanol precipitation. The RNA can be stored indefinitely at this stage. The second protocol is more rapid. It can be completed (i.e., to the ethanol precipitation) in less than 2 hr.

Literature Cited

Gilman, M.Z. and Chamberlin, M.J. 1983. Developmental and genetic regulation of *Bacillus subtilis* genes transcribed by sigma-28-RNA polymerase. *Cell* 35:285-293.

McKnight, G.S. 1978. The induction of ovalbumin and conalbumin mRNA by estrogen and progesterone in chick oviduct explant cultures. *Cell* 14:403-413.

Summers, W.C. 1970. A simple method for extraction of RNA from *E. coli* utilizing diethylpyrocarbonate. *Anal. Biochem.* 33:459-463.

Thomas, P.S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. U.S.A.* 77:5201-5205.

Key Reference

Summers, 1970. See above.

A brief but illuminating comparison of the basic methods for isolating RNA from E. coli.

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